



EXTRAORDINARY STABILITY OF IgE-BINDING *PARIETARIA* POLLEN ALLERGENS IN RELATION TO CHEMICALLY BOUND FLAVONOIDS

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Abstract—It is known that the skin-active and IgE-binding components in *Parietaria* pollen extracts are not restricted to the predominant protein allergens of M_r 12 000–15 000, but are present as well among the naturally occurring constituents of M_r < 10 000. Indeed, the IgE-binding *Parietaria* pollen components are quite heterogeneous, ranging from high- to low-molecular mass, whereby the IgE-binding epitopes display an unusual chemical stability. Furthermore, the pollen of *Parietaria* species demonstrably contain a high proportion of flavonoid pigments. Since these pollen grains cannot be collected entirely free from non-pollen plant parts, the usual allergenic extracts of *Parietaria* encompass both the polyphenolic substrate molecules and the enzyme polyphenoloxidase as ingredients for the oxidative generation of flavonol–protein conjugates during the extraction process. In the present work this is illustrated by spectroscopic analyses of the free and bound flavonoids in *Parietaria* pollen extracts, as well as of the peptide fragments produced from the allergenic proteins by enzymatic or chemical hydrolysis. None of these relatively harsh treatments had a significant effect on the IgE-binding properties of the allergenic (sub-)components, even though detectable proteins in isoelectric focusing and immunoblotting were lost. It is proposed that the extraordinary stability of IgE-binding *Parietaria* components over a wide molecular range may be attributed to chromophoric flavonoid side-chains as (parts of) the corresponding B-cell epitopes. © 1997 Elsevier Science Ltd. All rights reserved.

Key words: allergens, pollen, *Parietaria*, flavonoids.

INTRODUCTION

The *Parietaria* plant species are dicotyledonous weeds belonging to the family *Urticaceae*. *Parietaria* plants produce highly allergenic pollen grains, which may become released explosively from the elastic filaments of the anther and become wind-borne (Serafini, 1957; D'Amato *et al.*, 1992). However, owing to morphological characteristics, harvesting of the pollen granules for purposes of allergen studies invariably leads to contamination with non-pollen plant parts, i.e. from the anther. In most reports dealing with the isolation and identification of *Parietaria* pollen allergens, the proportion of green plant parts in the dry pollen powder is estimated at around 25% (Geraci *et al.*, 1985; Ford and Baldo, 1986; Cocchiara *et al.*, 1989). This practical issue must be taken into account when considering the published properties of the allergenic pollen proteins of *Parietaria* species.

While the predominant *Parietaria* allergens now identified as Par j 1 or Par o 1 are (glyco)proteins of M_r 12 000–15 000 under reducing conditions (Polo *et al.*, 1991;

Oreste *et al.*, 1991), separation studies have also demonstrated that the allergenic (skin test or IgE-binding) properties extend over a range of lower molecular mass fragments (Menegozzo *et al.*, 1976; Feo *et al.*, 1980; Falagiani *et al.*, 1985; Ruffili *et al.*, 1987). Furthermore, spectroscopic and chemical analyses have shown that both the “major” allergens of M_r 12 000–15 000 and the lower molecular IgE-binding fragments incorporate tightly bound chromophoric structures which have, so far, not been identified (Ruffili *et al.*, 1987; Ayuso *et al.*, 1988; Polo *et al.*, 1991). Some early chemical data were interpreted as indicating these chromophores to represent protein–sugar adducts of the Maillard-type (Geraci *et al.*, 1978).

The abundance of pigments even in dialysed aqueous extracts of defatted *Parietaria* pollen grains, readily detectable by the naked eye, raises the question of their precise chemical nature and possible contribution to allergenicity. In this context, it should be reiterated that the literature is quite consistent in describing an unusual resistance of *Parietaria* pollen allergens towards heat treatment and enzymatic digestion (Menegozzo *et al.*, 1976; Geraci *et al.*, 1978). Conversely, some authors consider the allergens as highly unstable due to their presumed ease of fragmentation (Bolzacchini *et al.*, 1991). It was the purpose of this investigation to examine these characteristics in more detail.

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MATERIALS AND METHODS

Preparation of pollen allergens and their fragments

Acetone-defatted and vacuum-dried pollen grains of *Parietaria judaica*, collected in Europe in 1994, were supplied by Biopol, Spokane, U.S.A. (lot Nos 51414, 51521, 51646; purity as stated: <0.1% foreign pollen and about 6% plant parts). The greenish powder was shipped in airtight containers containing a desiccant and was extracted twice with distilled water for 2 hr at room temperature immediately upon arrival. The combined and centrifuged aqueous extracts were dialysed in the cold from Visking casing (nominal cut-off $M_r > 10\,000$). Both the dialysate (LMW) and the non-dialysable components (E) of $M_r > 10\,000$ were dried by lyophilization. Chromophoric substances physically adsorbed to E were then eliminated by repeated dialysis of solutions of E in 0.01 N HCl, followed by neutralization and freeze-drying to give the depigmented proteins of $M_r > 10\,000$, E1. The pigments and other components thus removed at pH 2, representing 46% by weight of E, were also recovered by lyophilization after neutralization.

For enzymic fragmentation, a sample of E1 was dissolved in a suspension of pepsin immobilized on 4% cross-linked agarose (Sigma, St. Louis, U.S.A.) in a proportion of 2:1 w/w and the mixture in HCl at pH 2 was stirred for 6 hr at 37°C. The reaction was stopped by neutralization with NaOH and the enzyme particles were removed by filtration (0.2 µm). For eliminating salts and fragments of $M_r < 1000$, the digest was passed through a Minitan[®] tangential flow system equipped with a filter of $M_r\ 1000$. The retentate of $M_r > 1000$ was finally dialysed again by Minitan passage through a filter of $M_r\ 10\,000$, thus separating the endo-fragments of $M_r > 10\,000$, E2a, and the fragments of $M_r > 1000/<10\,000$, E2b. Digests E2a and E2b were dried by lyophilization, with a recovery from E1 of 57% and 26%, respectively.

An aliquot portion of E1 was hydrolyzed with 2 N HCl at 60°C for 2 hr, followed by neutralization, dialysis and fractionation, as described for the peptic digestion. Two new preparations were obtained, designated as E3a ($M_r > 10\,000$) and E3b ($M_r < 10\,000$), respectively. Finally, a basic hydrolysate was produced by incubation of E1 with 0.01 M NaOH for 6 hr at 37°C. After neutralization and removal of salts, the retentate portion of $M_r > 10\,000$ was dried by lyophilization to give preparation E4.

Normal (HMW-N) and depigmented (HMW-D) ($M_r > 10\,000$) allergenic preparations of *Lolium perenne* pollen (Biopol, Spokane, U.S.A., lot No. 51646, less than 2% plant parts as stated), as well as the digest of $M_r > 10\,000$ (fraction a, recovery from HMW-D 63% by weight) and the digest of $M_r < 10\,000$ (fraction b) obtained after treatment with pepsin (enzyme to protein ratio 1:5 w/w) were prepared as described for *Parietaria judaica*.

Progress of enzyme digestion was monitored by the liberation of free amino groups. At regular intervals during the process of enzymatic hydrolysis, 0.5 ml samples were taken from the pepsin/protein mixtures, adjusted to

a final concentration of 0.5 mg/ml in 4 N acetate buffer pH 5.5 and then heated for 15 min at 100°C with 50 µl of 2% ninhydrin in buffered methylcellosolve. After cooling, absorbance was read at 540 nm against the reagent blank.

The "major" allergen Par j 1 (Ayuso *et al.*, 1988; Cocchiara *et al.*, 1989) was isolated from the *Parietaria* E-preparation by a combination of procedures involving hydrophobic interaction on phenyl-Sepharose columns, followed by gel filtration on Sephadex G75. The final preparation was homogeneous in SDS-PAGE with respect to the characteristic IgE-binding Par j1 doublet (Cocchiara *et al.*, 1989; Polo *et al.*, 1991).

Ellagic acid was obtained from Sigma Chem. Corp. (St. Louis, MO, U.S.A.) and the flavonoid reference compounds were purchased from Carl Roth AG (Karlsruhe, Germany).

Specific IgE-antibody binding

Quantitative binding of specific IgE antibodies from human allergic serum was evaluated by enzyme immunoassay in inhibition format, in all cases using as solid phase the lyophilized material E ($M_r > 10\,000$) coupled to cellulose discs activated with cyanogen bromide (Ceska *et al.*, 1972). The serum pools in predetermined dilution (usually 1:4–1:8) were first incubated for 3 hr at room temperature with a series of concentrations of the soluble allergens, their hydrolytic derivatives or other modulators. In the second stage, these preincubated allergen-serum mixtures were each reacted with allergen-coated discs in order to capture residual unbound specific IgE. After washing with PBS-Tween (0.5%), monoclonal anti-IgE (Ingenasa, Madrid, Spain) labelled with horseradish peroxidase was added to the discs. After an additional 3 hr, the discs were washed repeatedly, whereupon the degree of specific IgE-binding was evaluated by colorimetric assay of bound peroxidase at 450 nm, using hydrogen peroxide as a substrate and tetramethylbenzidine for colour development. Results were expressed as percentage of maximum binding.

Isoelectric focusing and u.v.-absorption spectra

Isoelectric focusing in agarose gel containing 2.5% each of equal parts of Ampholines pH 3–5 and pH 5–8 was carried out following the manufacturer's instructions (BioRad, Richmond, U.S.A.). Lyophilized preparations were dissolved in phosphate-saline (PBS) buffer pH 7.4 and the electronic absorption spectra were recorded in a Philips u.v.-VIS Spectrophotometer in the range of 200–600 nm. A series of solutions at identical concentration in various buffers at different pH-values was made up for establishing the isosbestic point.

Sera

Blood serum samples from pollinosis patients sensitive by skin prick test to the pollen of *Parietaria judaica* or *Lolium perenne* were obtained from various hospitals in Spain. Samples were analysed for specific IgE antibodies

to each of these pollens (CAP-system, Pharmacia, Uppsala, Sweden) and the high-titered samples were organized into two separate pools, one positive for *Parietaria*-IgE and the other for *Lolium*-IgE. Serum pools were stored frozen in small aliquots at -20°C until use.

RESULTS

Enzymatic hydrolysis

During introductory experiments it was established that the allergenic proteins in various pollen species are more susceptible to digestion by pepsin than by bovine trypsin. Also, there were notable differences in the rate of peptic hydrolysis among the proteins of $M_r > 10\,000$ from different pollen species. For example, the *Lolium* allergenic pollen proteins were readily cleaved by pepsin, whereas the *Parietaria* components of $M_r > 10\,000$ proved much more resistant, as shown by the slow rate of appearance of ninhydrin-reactive material in Fig. 1. Separate experiments using TLC confirmed only minimal release of ninhydrin-positive amino acids or peptides, even with hot 2 N HCl. From these and other data, a hydrolysis period of 6 hr was deemed adequate for further enzymatic fragmentation studies (Materials and Methods).

Isoelectric focusing

A comparison was made of the protein composition of the different *Parietaria* pollen preparations by isoelectric focusing in agarose gel. Figure 2 shows the electrophoretic focusing patterns observed with the *Parietaria* preparations and hydrolysates under study.

The IEF patterns of Fig. 2 demonstrate that the desorption in acid medium of electrostatically held pigments does not significantly affect the overall protein distribution (Fig. 2, lane 3) nor the IgE binding (Fig. 3(A)), indicating acid stability at pH 2. Treatment with HCl at pH 2 in the presence of pepsin, however, caused a virtually complete loss of all detectable protein bands,

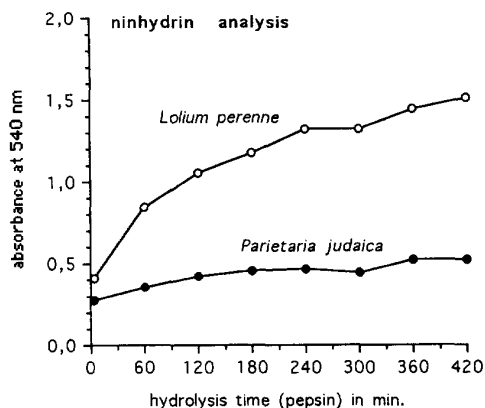


Fig. 1. Kinetics of peptide/amino acid liberation during the digestion of Par-HMW-D with immobilized pepsin at 37°C , as determined by colour development with ninhydrin (Materials and Methods).

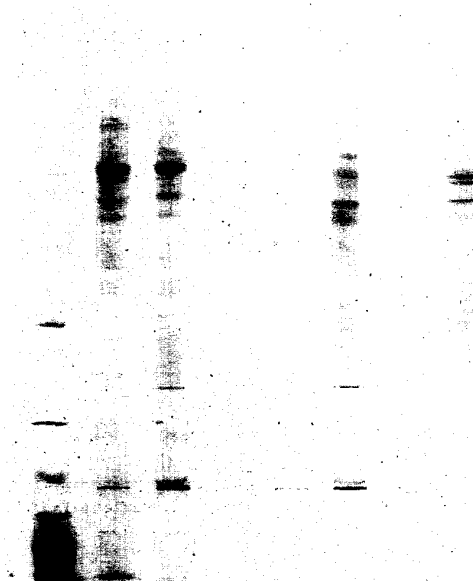


Fig. 2. Analytical isoelectric focusing in agarose gel in the pH range 3–8 of the various *Parietaria* protein fractions and fragments investigated. Application: $2.5\ \mu\text{l}$ 4% solution of lyophilized material E (lane 2), E1 (lane 3), E2a (lane 4), E2b (lane 5), E3a (lane 6), E3b (lane 7) and E4 (lane 8). IEF standard proteins (Bio-Rad) in lane 1. Lanes are numbered left to right. Stain: Coomassie Brilliant Blue. Anode on top.

both in the cleavage products of $M_r > 10\,000$ (E2a, lane 4) and in the fragments of $M_r < 10\,000$ (E2b, lane 5). Presumably, the fragmented material is not amphophilic or is being washed out of the gel, even though both fractions remained virtually unaltered in IgE-binding (Fig. 3(A)). Treatment of the *Parietaria* proteins with hot and concentrated HCl neither impeded protein detection in IEF (fraction E3a, Fig. 2, lane 6), nor IgE-binding (Fig. 3(B)). The low molecular weight material E3b split off with strong acid was undetectable in IEF by protein staining (Fig. 2, lane 7), but continued to display full IgE-binding potency (Fig. 3(B)).

Only prolonged treatment of the *Parietaria* proteins with alkali at pH 12 caused a slight loss of IgE-binding (Fig. 3(C)), although the effect on the IEF pattern was minimal (fraction E4, Fig. 2, lane 8).

IgE antibody binding

Within the accuracy limits of the inhibition assay of specific IgE antibody binding, no outstanding potency differences were observed between the allergenic preparations, as demonstrated in the dose-response graphs of Fig. 3(A–C). On a weight basis, the ratios for 50% inhibition of IgE-binding by the *Parietaria* preparations were E:E1:E2a:E2b:E3a:E3b:E4 = 1.2:1.0:0.7:1.9:0.9:1.1:3.0, whereby the lowest figures represent the highest IgE-binding potency. Hence, the removal of adsorbed pigments from the proteins E ($M_r > 10\,000$), in fact, slightly increased the IgE-binding potential (E1), while the IgE-binding power of the various high- and low-molecular fractions isolated after pepsin hydrolysis or hot acid treat-

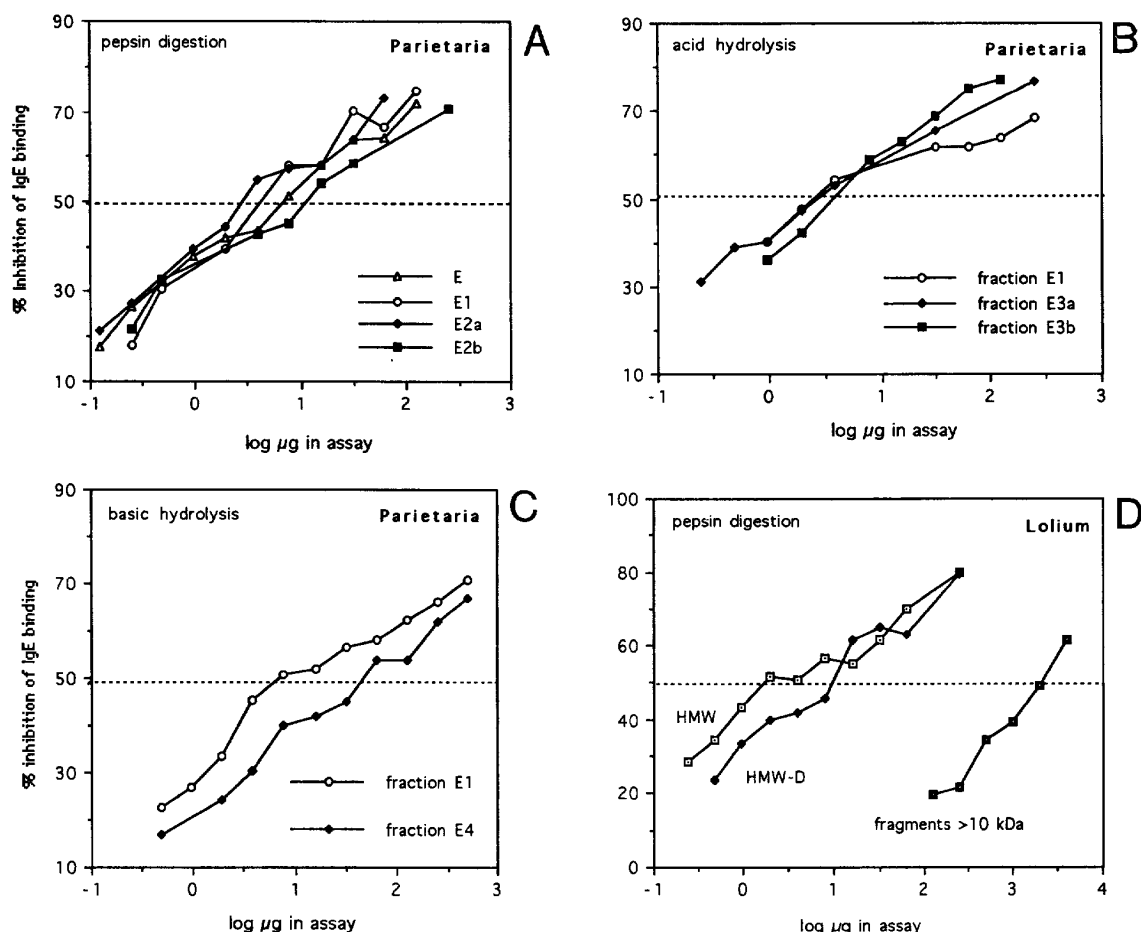


Fig. 3. Potency assay by inhibition of specific IgE antibody binding from the serum of pollen-sensitive patients, using the allergenic material obtained by: (A) aqueous extraction (E), depigmentation (E1) and peptic digestion (E2a and E2b); (B) acid hydrolysis (E3a, E3b); and (C) basic hydrolysis (E4). (D) The dose-response curves for the binding of specific IgE to *Lolium perenne* by the normal protein allergens HMW-N, the depigmented derivative HMW-D and the peptic fragments of $M_r > 10\,000$ (see Materials and Methods). The point of 50% inhibition is at the intersection with the dotted lines.

The ratios of potencies in terms of doses for 50% inhibition are given in the text.

ment showed no significant change relative to the parent material, E1. Only alkaline conditions caused a minimal reduction of potency by a factor of 3. The original LMW-components were less active by a factor of 6–10 (not shown). IgE-inhibition by all of these *Parietaria* protein derivatives was allergen-specific, because inhibition was not observed with *Lolium perenne* or *Betula alba* allergens, in the heterologous system comprising *Parietaria*-positive serum and discs coated with *Parietaria* preparation E.

With the *Lolium perenne* allergens (Fig. 3(D)), the ratio of specific IgE-binding potencies was found to be HMW-N:HMW-D:peptic fragments of $M_r > 10\,000$ (fraction a)=1:2.4:500, while the fragments of $M_r < 10\,000$ (fraction b) were at least 1000 times less active than HMW-N (data not shown). The IgE-binding epitopes of the *Lolium perenne* proteins are, therefore, lost or become inaccessible by pepsin hydrolysis, in sharp contrast to the observations with the *Parietaria* allergens.

No RAST-inhibition was observed with the Factor XII activator ellagic acid in an extended dose range up to

20 mg/ml. Similarly, the flavonoids naringenin-7-glucoside, apigenin-7-glucoside, quercitrin dihydrate and quercetin dihydrate in the same dose range did not inhibit specific anti-*Parietaria* IgE binding.

Ultraviolet-spectroscopic analysis

In an attempt to understand the remarkable resistance of the IgE-binding epitopes, we focused attention on a conspicuous common factor among the various *Parietaria* (but not the *Lolium*) preparations, namely the prominent distribution of chromophores remaining unaffected by physical, chemical or enzymic manipulation. Spectroscopic analysis was done on each of the preparations, including the isolated major allergen Par j 1. The u.v.-absorption spectra are shown in Fig. 4.

Comparison of the electronic spectra with published data and with those of pure flavonoids confirmed that the prominent 355 nm peak (at pH 2 or 7, showing a bathochromic shift towards 395 nm at pH 9–10) was

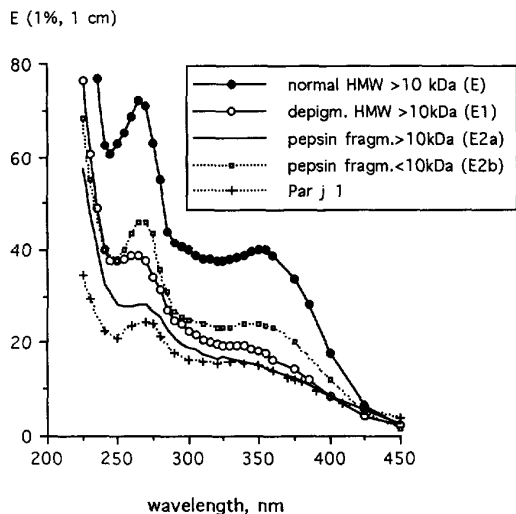


Fig. 4. Ultraviolet-absorption spectra at pH 7 of *Parietaria* E, E1 and pepsin fragments E2a and E2b. Included is the spectrum of the "major" allergen Par j 1.

present in all the *Parietaria* preparations. These characteristics are compatible with polyhydroxylic flavonol derivatives of the quercetin type. Addition of FeCl_3 to dilute solutions of the *Parietaria* preparations indeed produced a characteristic green colour, shifting the spectrum well into the visible region, indicating an *o*-biphenolic configuration on the flavonoid core structure. However, the water-soluble free (LMW) flavonol-glycosides of $M_r < 10\,000$, exposing similar spectroscopic characteristics (Fig. 5), had only low IgE-binding potency (data not shown).

As illustrated with the protein-free dialysable LMW constituents in Fig. 5, the u.v.-absorption spectra of the *Parietaria* preparations feature an isosbestic point similar as observed with quercetin and some other flavonols.

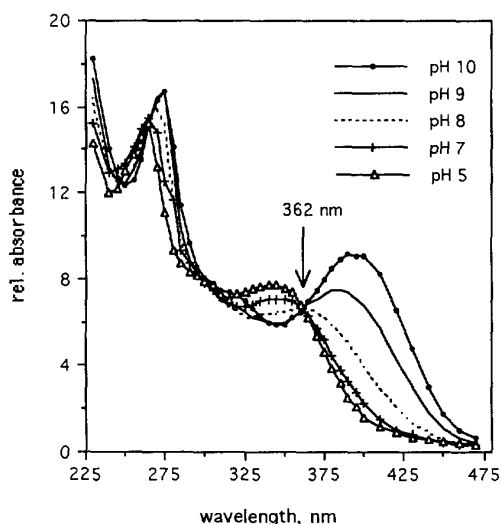


Fig. 5. Ultraviolet-absorption spectra of *Parietaria* LMW components ($M_r < 10\,000$) in different buffer solutions at the pH-values as indicated, showing the isosbestic point at 362 nm typical of flavonols of the quercetin type.

Although preliminary analyses by thin-layer chromatography indicate considerable heterogeneity of the constituent flavonoids within and between the various *Parietaria* fractions, the overall u.v.-spectra in all cases were largely determined by the dominant quercetin-type flavonol(s) revealed in Figs 4 and 5. The absorption maximum (for peak II) at 395–400 nm at pH 10, and the isosbestic point at 362 nm are, indeed, typical of flavonol (3)-glycosides (in water) or their aglycones (in organic solvents), more particularly of 3',4'-biphenolic flavonols of the quercitrin or quercetin class (Mabry *et al.*, 1970).

Alkaline conditions, as well as acid treatment with hot 2 N HCl, would hydrolyse the carbohydrate segment of the flavonol-glycosides. It is likely, therefore, that the flavonols detected among the IgE-binding *Parietaria* protein derivatives ($M_r > 10\,000$) E3a, E3b and E4 are in the form of their aglycones chemically bound to amino- or sulfhydryl-groups of amino acids or peptides.

DISCUSSION

The results of this investigation are concordant with previous reports on the remarkable thermal stability and protease resistance of the IgE-binding epitopes of natural *Parietaria* pollen allergens (Menegozzo *et al.*, 1976; Geraci *et al.*, 1978; Feo *et al.*, 1980; Polo *et al.*, 1991). Even though some hydrolysis did occur with pepsin (Fig. 1), the enzymatic fragments nonetheless fully retained the IgE-binding potency (Fig. 3). In agreement with the deglycosylation experiments of Polo *et al.* (1991), Mucci *et al.* (1992) and others, deliberate hydrolysis with hot concentrated acid or alkali had no effect on specific IgE antibody binding, thus confirming that carbohydrates are not involved in the B-cell epitope structures. Our experiments further corroborate that the *Parietaria* allergens may present themselves as a family of fragments of almost equal IgE-binding potency (Menegozzo *et al.*, 1976; Feo *et al.*, 1980; Falagiani *et al.*, 1985; Ruffili *et al.*, 1987), even though such fragments may escape detection in electrophoresis (Fig. 2) or immunoblotting (not shown). In contrast, the IgE-binding protein allergens of *Lolium perenne* pollen proved quite susceptible to pepsin digestion and loss of IgE-binding epitopes (Fig. 3(D)).

The observation that the IgE-binding potencies of the ($M_r > 10\,000$) proteins E, E1 and the pepsin treated fraction E2a remained virtually unchanged on a weight basis, despite the elimination of a considerable proportion of substance during their preparation (Materials and Methods), denotes that the desorbed or hydrolysable fragments of $M_r < 10\,000$ contribute appreciably to the total IgE-binding potential of full *Parietaria* pollen extracts. This must be taken to mean that the IgE-binding epitopes disseminate over a wide and diffuse range of high- and low-molecular mass components without discernible effect on the antibody-binding properties. It may be assumed, therefore, that it is the density and/or the intermolecular distance of the epitopes on the (poly) peptides in the extract which determines the IgE-binding potency of any given (sub-)fraction. A similar conclusion has been reached in relation to *in vitro* complement acti-

vation by *Parietaria* pollen allergens and their fragments (Berrens and De la Cuadra López, in press).

The spectroscopic analyses suggest that the chromophoric components previously detected among the *Parietaria* pollen allergens, including the "major" allergen Par j 1, largely pertain to the category of the flavonoid pigments (Geraci *et al.*, 1978; Ruffili *et al.*, 1987; Ayuso *et al.*, 1988; Polo *et al.*, 1991). Tentatively, the spectroscopic data indicate that the predominant pigments may comprise polyhydroxylic flavonol compounds featuring a (3',4')-*o*-biphenolic configuration on the phenyl residue, most probably conjugated as aglycones to (poly)peptides through the B-ring of the flavonoid central structure. Such conjugates with free protein amino- or sulfhydryl groups may become generated under oxidative conditions by nucleophilic attack at positions 2' or 5' on the phenyl ring (Hagerman and Kluger, 1986).

Though nonenzymic "browning" reactions between proteins and flavonoids have been described (Igarashi *et al.*, 1978), the presence of plant polyphenoloxidases would greatly enhance the coupling reaction. As mentioned in the Introduction, the pollen of *Parietaria* spp. cannot easily be collected free of plant residues. Pure pollen does not contain the enzyme polyphenoloxidase, whereas other plant parts do (Stanley and Linskens, 1974). Together with the high content of yellow pigments in *Parietaria* pollen (Serafini, 1957; Geraci *et al.*, 1978; Ayuso *et al.*, 1988; Polo *et al.*, 1991), the conditions during aqueous extraction would therefore favour the oxidative generation of polycondensates of *o*-quinones or flavonoid(-aglycones) complexed with amino acids, peptides or proteins, ranging from low to high molecular mass. However, condensed tannins comprising solely flavonoid (co-)polymers and forming tight physical complexes with proteins might arise as well. The known properties of the tannins and conjugated flavonoids provide a satisfactory explanation for the remarkable chemical stability and protease resistance of the *Parietaria* pollen allergens (Hagerman and Kluger, 1986), as well as for the light absorption and fluorescence characteristics (Ayuso *et al.*, 1988; Polo *et al.*, 1991) and the oxygen-scavenging properties (Serafini, 1957; Geraci *et al.*, 1978).

It has been shown in the past that isolated free flavonoids or their glycosides do not possess allergenic (skin test) activity (Moore and Moore, 1931; Stevens *et al.*, 1951). In our studies, we did not observe inhibition of specific IgE-binding by some selected free flavonoid (-glycosides). Given the abundance of fluorescent spots on thin-layer chromatograms of the *Parietaria* preparations (not shown) and the distinct physico-chemical behaviour of peptide-bound flavonoids, the precise chemical identification of possible haptenic flavonoids cannot easily be achieved, however. Nevertheless, the accumulated evidence does suggest the possibility that flavonoids conjugated in specific structural fashion to side-chains of (poly)peptide carriers might, indeed, as haptens contribute to allergenicity and epitope structure, as inferred before in the case of ragweed and rye pollen (Stevens *et al.*, 1951; Goldfarb *et al.*, 1954; Scheibe and Loewe, 1969) and the low-sized timothy pollen allergens (Rockwell, 1943; Lee *et al.*, 1971; Malley *et al.*, 1975).

It is evident that more studies are needed on the presumed haptenic nature of the conjugated flavonoids, especially in relation to the structure of the (IgE) B-cell epitope(s) deduced from studies with recombinant Par j 1 (Costa *et al.*, 1994; Scotto D'Abusco *et al.*, 1996). Remarkably, the recently proposed sequence of a dominant linear peptide epitope includes a cysteine residue (Scotto D'Abusco *et al.*, 1996), whereas no free thiol groups were detected upon amino acid analysis of native Par j 1 (Polo *et al.*, 1991). The discrepancy might be explained by assuming that in the native situation the sulfhydryl side-chain of the deduced peptide epitope might be blocked by a chemical hapten as part of the IgE-binding antigenic determinant.

The chemical and enzymatic resistance and the retention of activity even on small fragments represents a dilemma in allergen standardization and control because, whenever the requisite electrophoretic analyses fail to reveal any protein bands, not even the "major" allergen Par j 1, the extracted material may still retain authentic IgE-binding potency. The case of *Parietaria* pollen, therefore, underlines the need for a critical review of inconsistencies among the currently authorized methodologies for allergen standardization.

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